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DNA Vaccine to Western Equine Encephalitis Virus

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Abstract

Previously, the complete genome of western equine encephalitis virus had been cloned and sequenced. This paper describes mammalian expression vectors pCXH-3 and pVHX-6, in which expression of the structural genes of western equine encephalitis virus have been placed under the control of the mammalian CMV promoter. When pCXH-3 or pVHX-6 is expressed using a cell-free transcription/translation system, *in vitro*, authentic structural proteins of western equine encephalitis virus are produced as verified by reactivity with monoclonal antibodies developed to western equine encephalitis virus. These vectors can also be complexed with liposomes and administered to mammalian cell culture. The viral envelope proteins were functionally expressed, as determined by histochemical staining with monoclonal antibodies which recognize WEE antigens. In addition, when pCXH-3 or pVHX-6 is administered intraepidermally and intramuscularly to mice, a protective immune response is induced. Immunized mice had a significantly reduced risk of infection, against a subsequent intranasal challenge with western equine encephalitis virus. Development of a DNA vaccine to western equine encephalitis virus is promising. In a similar manner, DNA vaccines to related alphaviruses (Venezuelan and eastern equine encephalitis viruses) could also be developed.

Résumé

Le génome complet du virus de l'encéphalite équine de l'Ouest (WEE) avait été cloné et mis en séquence, auparavant. Cet article décrit les vecteurs d'expression génétique mammifère pCXH-3 et pVHX-6, dans lesquels les gènes de structure du virus de l'encéphalite équine de l'Ouest ont été placés sous le contrôle du promoteur mammifère CMV. Quand l'expression pCXH-3 ou pVHX-6 est réalisée *in vitro*, utilisant un système acellulaire de transcription et de traduction, on réussit à produire des protéines de structure authentiques du virus de l'encéphalite équine de l'Ouest tel que vérifié par la réactivité avec les anticorps monoclonaux développés pour le virus de l'encéphalite équine de l'Ouest. Ces vecteurs peuvent être aussi introduits dans des liposomes et administrés à une culture de cellule mammifère. Les protéines de l'enveloppe virale ont été exprimées selon leurs fonctions, tel que déterminées par la coloration histochimique d'anticorps monoclonaux reconnaissant les antigènes WEE. De plus, on induit une réaction immunitaire protectrice quand pCXH-3 or pVHX-6 est administré à des souris de manière intraépidermique ou intramusculaire. Le risque d'infection des souris immunisées diminue de manière significative contre les expositions intra-nasales de contrôle au virus de l'encéphalite équine de l'Ouest. Le développement d'un vaccin ADN contre le virus de l'encéphalite équine de l'Ouest est prometteur. Les vaccins ADN contre les arbovirus A (les virus de l'encéphalite équine vénézuélienne et de l'Est) pourraient être aussi mis au point de la même manière.

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Executive summary

The encephalytic alphaviruses include western, eastern and Venezuelan equine encephalitis viruses (WEE, EEE and VEE, respectively). These range in pathogenesis from incapacitation for VEE to lethal encephalitis, with a 3-7% case fatality rate for WEE and a 40% case fatality rate for EEE. VEE is highly infectious, with as little as 1-2 viral particles able to cause an infection. All are aerosol transmissible and pose a potent BW threat. Currently, no medical countermeasures are available in terms of either vaccines or antivirals. A live attenuated vaccine for VEE is under development at USAMRIID. Polyriboinosinic-polyribocytidylic acid poly-L-lysine carboxymethylcellulose (poly ICLC) demonstrates promise in protection against WEE infections, if given 24 hr pre-exposure (Wong *et al.*, 2001a).

Previously, the 26S region of the genome of western equine encephalitis virus (which encodes the structural genes) had been cloned and sequenced (Schmaltz *et al.*, 1998; Netolitzky *et al.*, 2000). This paper describes mammalian expression vectors pCXH-3 and pVHX-6, in which expression of the structural genes of western equine encephalitis virus have been placed under the control of the mammalian CMV promoter. When pCXH-3 or pVHX-6 was expressed using a cell-free transcription/translation system *in vitro*, authentic structural proteins of western equine encephalitis virus were produced as verified by reactivity with monoclonal antibodies, previously developed to western equine encephalitis virus (Long *et al.*, 2001). These vectors can also be complexed with liposomes and administered to mammalian cell culture. The viral envelope proteins were functionally expressed, as determined by histochemical staining with monoclonal antibodies which recognize WEE antigens.

An intranasal infectivity model was developed using the Fleming strain of WEE, which was 100% lethal to BALB\c mice. Strain differences in virulence were observed in this infectivity model. When pCXH-3 or pVHX-6 was administered *in vivo* to mice, intraepidermally (ballistically) and intramuscularly, a protective immune response was induced. Immunized mice had a significantly reduced risk of infection against a subsequent intranasal challenge with WEE.

Development of a DNA vaccine to western equine encephalitis virus is very promising. Improvements can be made to increase the effectiveness of the vaccine, including the use of encapsulation to reduce the number of doses required, and to engineer the structural genes from a second strain of WEE with the aim of achieving better cross-protection. In a similar manner, DNA vaccines to related alphaviruses (Venezuelan and eastern equine encephalitis viruses) could also be developed.

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Sommaire

Les arbovirus A ancéphalytiques incluent les virus de l'encéphalite équine de l'Ouest, de l'Est et vénézuélienne (WEE, EEE et VEE respectivement). Les pathogenèses varient de l'incapacité pour le VEE, à l'encéphalite mortelle ayant un taux de mortalité variant de 3 à 7 % pour le WEE et un taux de mortalité de 40% pour le virus EEE. Le VEE est extrêmement infectieux; 1 à 2 particules virales peuvent, à elles seules, causer une infection. Ces virus sont tous transmissibles par aérosols et posent une menace grave de Guerre biologique. Actuellement, il n'existe pas de contre-mesures médicales disponibles en termes de vaccins ou d'antiviraux. Un vaccin vivant atténué de VEE est en cours de mise au point à l'agence USAMRIID. Le poly ICLC (carboxyméthylcellulose acide poly-L-lysine polyriboinosinique-polyribocytidylique) se montre prometteur pour protéger contre les infections VEE s'il existe une pré-exposition de 24 heures (Wong *et al.*, 2001a).

La région 26S du génome du virus de l'encéphalite équine de l'Ouest (qui encode les gènes de structure) a été clonée et mise en séquence auparavant. (Schmaltz *et al.*, 1998; Netolitzky *et al.*, 2000). Cet article décrit les vecteurs d'expression génétique mammifère pCXH-3 et pVHX-6, dans lesquels les gènes de structure du virus de l'encéphalite équine de l'Ouest ont été placés sous le contrôle du promoteur mammifère CMV. Quand l'expression pCXH-3 ou pVHX-6 est réalisée *in vitro*, utilisant un système acellulaire de transcription et de traduction, on réussit à produire des protéines de structure du virus de l'encéphalite équine de l'Ouest tel que vérifié par la réactivité contre les anticorps monoclonaux développés, au virus de l'encéphalite équine de l'Ouest (Long *et al.*, 2001). Ces vecteurs peuvent être aussi introduits dans des liposomes et administrés à une culture de cellule mammifère. Les protéines de l'enveloppe virale ont été exprimées selon leurs fonctions, tel que déterminées par la coloration histochimique d'anticorps monoclonaux reconnaissant les antigènes WEE.

Un modèle d'infectivité intra-nasal a été mis au point en utilisant la souche Fleming du virus WEE qui est 100% mortel pour les souris BALB\c. On a observé des différences de virulence selon les souches dans ce modèle d'infectivité. On a induit une réaction immunitaire protectrice, quand pCXH-3 or pVHX-6 a été administré *in vivo* à des souris, de manière intraépidermique (balistique) et intramusculaire. Le risque d'infection des souris immunisées diminue de manière significative contre les expositions intra-nasales de contrôle contre le virus WEE.

La mise au point d'un vaccin ADN contre le virus de l'encéphalite équine de l'Ouest est très prometteuse. L'apport d'améliorations pourrait augmenter l'efficacité du vaccin; l'encapsulation réduirait le nombre de doses requises, la création des gènes de structure à partir d'une deuxième souche de WEE permettrait une meilleure protection. Les vaccins ADN contre les arbovirus A (les virus de l'encéphalite équine vénézuélienne et de l'Est) pourraient être aussi mis au point de la même manière.

Nagata, L.P., Masri, S.A., Long, M.C., Schmaltz, F.L., Rayner, G.A. and Wong, J.P. 2001. DNA Vaccine to Western Equine Encephalitis Virus. TR-2002- 023. Defence Research Establishment Suffield.

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Acknowledgements

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Introduction

The alphaviruses are a group of about 27 enveloped viruses with a positive sense, single-stranded RNA genome (Calisher *et al.*, 1980; Strauss and Strauss, 1988). The alphavirus studied in this report, western equine encephalitis virus (WEE), is a member of the WEE antigenic complex and is serologically related to the Sindbis (SIN), Highlands J, Fort Morgan, Buggy Creek, and Aura viruses (Calisher & Karabatsos, 1988; Calisher *et al.*, 1988). WEE is endemic in western North America and strains/varieties have been isolated from Argentina, Brazil and the former Soviet Union (Johnson and Peters, 1996; Weaver *et al.*, 1997). In nature, WEE is transmitted from its amplifying hosts or reservoir in wild birds, to humans and horses, by mosquitoes (*Culex tarsalis* being the principal vector). While the endemic cycle has resulted in a limited number of human infections in recent years, major epidemics of WEE have been recorded in the past. The most extensive epidemic, including 3,336 recognized human cases and 300,000 cases of encephalitis in horses and mules, occurred in the western United States and Canada in 1941 (Reisen & Monath, 1988; Johnson and Peters, 1996).

All alphaviruses share a number of structural, sequence, and functional similarities, including a genome with two polyprotein gene clusters (reviewed in Strauss & Strauss, 1994; Schlesinger & Schlesinger1996). The genomic organization of these viruses is conserved, with the nonstructural proteins translated directly from the 5' two-thirds of the genomic RNA. A subgenomic positive-stranded RNA (the 26S RNA) is identical to the 3' one-third of the genomic RNA and serves as the translational template for the structural proteins (capsid, E3, E2, 6K and E1).

The relationship of different WEE isolates to each other has been demonstrated using neutralization tests (Calisher *et al.*, 1988). Additionally, several strains of WEE were typed by oligonucleotide fingerprinting and found to have greater than 90 % nucleotide homology (Trent & Grant, 1980). The N-terminal sequences of the nucleocapsid and the E1 and E2 glycoproteins have been determined by Edman degradation; the E1 and E2 proteins were found to have 82 % and 71 % homology, respectively, to SIN (Bell *et al.*, 1983). Hahn *et al.* (1988) sequenced the 26S region of WEE strain BFS1703. They proposed that WEE originated as a hybrid virus, formed by recombination of an eastern equine encephalitis virus (EEE) and a Sindbis-like virus, most likely during a co-infection event. They suggested that two crossover events occurred, one within the E3 gene, the other within the 3' nontranslated terminal region (NTR), resulting in a virus whose nonstructural domain, intragenic region, and capsid protein are similar to EEE, with envelope proteins showing homology to SIN. Short regions within the NSP4 gene and the E1 protein/3' NTR have been determined for many WEE strains, allowing a preliminary assessment of the nucleic acid phylogenetic relationships within the WEE antigenic complex (Weaver *et al.*, 1997). Taken together, the data suggests that a vaccine derived from a single WEE strain should be able to cross-protect against the majority of the WEE isolates encountered in nature.

In terms of therapy or prophylaxis to WEE, there are very limited biologicals under study. An inactivated vaccine to WEE is under investigational new drug status. The vaccine uses formalin-inactivation of cell culture supernatants from WEE-infected tissue culture. It requires a minimum of three doses, yearly monitoring of antibody titer and possible boosters.

Its effectiveness against protection of an aerosol challenge of WEE has yet to be established. A WEE live attenuated vaccine is under development, based on mutagenesis of an infectious clone, as is currently underway for a Venezuelan equine encephalitis virus (VEE) vaccine. The immune modulator, poly ICLC, demonstrated protection to WEE infections if given 24 hr pre-exposure, in a mouse protection model (Wong *et al.*, 2001a)

The area of DNA immunization is relatively new, and has been extensively reviewed (Robinson *et al*, 1995; Hassett and Whitton, 1996; Donnelly *et al.*, 1997). Similar to live, attenuated vaccines, DNA vaccines are known to stimulate both humoral and cellular immune responses (Pardoll and Backering, 1997; McCuskie and Davies, 1999). Much of the focus has been on methods to deliver and efficiently express the cloned products. Intramuscular (IM) administration of DNA has been one of the original methods used (Wolff *et al.*, 1990). A second method uses ballistic (intraepidermal) delivery of DNA coated onto gold particles. High pressure helium gas is used to propel the particles into the epidermis and dermis of animals (Prayaga *et al.*, 1995). Furthermore, liposomal encapsulated DNA has been used to efficiently deliver DNA to protect against influenza A infection (Wong *et al.*, 2001b).

Our interest in WEE includes the development of subunit vaccines to WEE. Previously, we had cloned and sequenced the 26S region from WEE strain 71V1658 (Schmaltz *et al.*, 1997, Netolitzky *et al.*, 2000). The mammalian expression constructs, pCXH-3 and pVHX-6, were shown to functionally produce the WEE structural proteins *in vitro*. They were subsequently used in DNA immunization studies in a mouse model for protection against intranasal administered WEE.

Materials and Methods

Virus Culture and Purification

Tissue culture was maintained in accordance with established methods (Bird & Forrester, 1981). Minimal essential media containing 5 % fetal calf serum (5% DMEM) was used to grow Vero (CCL-81) and Chinese hamster ovary (CHO) K1 (CCL 61) cells obtained from American Type Culture Collections (Mannanas, VA). A 10 % suckling mouse brain suspension of WEE strain 71V-1658 was kindly provided by Dr. Nick Karabatsos, Centers for Disease Control, Fort Collins, CO; WEE Fleming and California strains were purchased from ATCC (Mannanas, VA); WEE B11 and CBA87 strains were kindly provided by Dr. George Ludwig, United States Army Medical Research Institute of Infectious Disease (Frederick, MD). Seed stocks of WEE strains were made by inoculation of Vero cells with virus suspensions at a multiplicity of infection (MOI) of less than 0.1. The supernatants were aliquotted and stored at -70°C.

Nucleic Acid Manipulation

Manipulation of DNA followed established procedures (Sambrook *et al.*, 1989; Ausubel *et al.*, 1995). Rapid plasmid preparations were made using the Wizard™ plasmid purification kit (Promega, Madison, WI) or Qiagen miniprep kits (Chatworth, CA). Large-scale plasmid preparations used the alkali lysis protocol as modified by Qiagen (Chatsworth, CA) to remove LPS. DNA sequencing oligonucleotide primer design was guided by information from WEE strain BFS1703 (Hahn *et al.*, 1988). Oligonucleotides were synthesized and gel purified either at the Regional DNA Synthesis Laboratory (Calgary, Alberta), or on a Beckman Oligo 1000 DNA synthesizer.

Automated DNA sequencing was performed using the ABI Prism Dye Terminator Cycle Sequencing or Big-Dye™ Terminator Cycle Sequencing kits of plasmid templates according to the manufacturer's instructions (PE-Applied Biosystems, Foster City, CA). Sequencing reactions were purified on Centri-Sep™ columns (Princeton Separations, Adelphia, NJ), dried and analyzed on an ABI 373 or 310 automated sequencer. Sequence traces generated were edited manually and assembled using the Seqman component of the Lasergene DNA analysis software (DNAStar, Madison, WI). DNA analysis was performed using Lasergene DNA analysis software, and plasmid maps generated using the Gene Construction Kit (Textco, West Lebanon, NH).

Construction of pCXH-3

The construction of the clone, pcDWXH-7, encoding the complete 26S region of WEE 71V-1658 has been described (Schmaltz *et al.*, 1997, Netolitzky *et al.*, 2000). The structural gene insert from pcDWXH-7 was cloned into the mammalian expression vector, pCI (Promega, Madison, WI). The pcDWXH-7 plasmid was first linearized using *Hind*III, followed by a Klenow fragment reaction to fill in the 5' overhang. The insert was then excised using *Xba*I,

gel purified and ligated into the *XbaI/SmaI* digested pCI vector. The isolated recombinant plasmid, pCXH-3, was characterized as having the correct insert by restriction mapping.

Construction of pVHX-6

The clone, pcDWXH-7, encoding the complete 26S region of WEE 71V-1658, was digested with *Sac*I, and the insert religated in the opposite orientation. The isolate, pcDWHX-45, contained the complete 26S genome of WEE, with the reverse order of cloning sites outside the two *Sac* I sites (*Hind*III on the 5' end and *Xba*I on the 3' end). The WEE 26S gene segment was excised from pcDWHX-45, and cloned into the *Hind*III and *Xba*I sites of the mammalian expression vector, pVAX (Invitrogen, La Jolla, CA). After tranformation into *E. coli* DH10α (Life Sciences, Burlington, ON) and screening of inserts by restriction analysis, a resulting isolate, pVHX-6 was identified.

Expression of the Structural Genes of WEE

One-step *in vitro* transcription and translation reactions using the TNT™ coupled system (Promega Corporation, Madison, WI) was used to express the gene products from the insert of both pCHX-3 and pVHX-6 from the upstream T7 promoter. The RNA was translated in the presence of [^{35}S]methionine to produce radiolabeled WEE proteins, which were further processed with canine pancreatic microsomal membranes. All components of the *in vitro* transcription and translation reactions were incubated together for 90 min at 30°C. Results were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE or radioimmunopricipitation.

In a second method, pCXH-3 expression vector was transfected into Vero or CHO K1 cells using the cationic lipid, Lipofectamine™ (Gibco/BRL, Burlington, ON). Briefly, Vero or CHO K1 cells were grown to 30-50 % confluency in Costar 6-well plates. The monolayers were transfected with pCXH-3 in accordance with the manufacturer's directions, for a period of 5 hrs, followed by a further 29 hr incubation after the addition of 5% DMEM. The monolayers were fixed in methanol:acetone (1:1) for 5 min and washed with PBS containing 0.1 %(v/v) Tween 20 and 3 % BSA (PBS-TB). The cells were incubated 45 min at 37 °C with a 1/100 dilution (in PBS-TB) of concentrated cell supernatant from hybridoma cell lines expressing monoclonal antibodies to the WEE E1 (clone 11D2) or E2 (clone 3F3) proteins (Long *et al.*, 2000b), followed by washing with PBS-TB. Monolayers were incubated with a 1/4000 dilution of goat anti-mouse IgG/IgM (H & L) horse radish peroxidase conjugate (Caltag, So. San Francisco, CA) for 45 min at 37 °C. After washing with PBS-T, 2 mL of TruBlue™ HRP substrate (Kirkegaard & Perry Laboratories, Gaitherburg, MD) was added, and plates were incubated a further 30 min at RT, followed by microscopic examination.

Administration of DNA or Inactivated Virus

DNA solutions or an inactivated WEE virus vaccine in PBS, were administered to the mice by ballistic or IM routes. For IM route of administration, a 27 g needle was used to deliver 50 µg of DNA (pCXH-3 or pCI - negative control) or 50 µL of inactivated WEE vaccine (SALK WEE inactivated vaccine). The final volume of inoculum used was 100 µl, diluted in PBS.

Fifty μL was administered IM to each of the hind leg muscles of a mouse. When boosters were given, they were administered 14-28 days apart. For ballistic administration, mice were shaved in the abdominal area with electric hair clippers. The mouse was subjected to ballistic delivery of DNA coated onto gold particles following the manufacturer's standard specifications. The Helios Gene Gun (Biorad, Mississauga, ON) was used as directed, at a pressure setting of 400 psi. Mice were given 1.25 μg DNA and 0.5 mg gold, 1 μm diameter, per shot, and up to three shots at one time. Boosters were given 14-28 days apart. The mice were challenged 14-28 days after the final booster.

Mouse Infectivity with WEE

Female BALB/c mice, 17-25 g, were obtained from the mouse breeding colony at Defence Research Establishment Suffield (DRES), with the original breeding pairs purchased from Charles River Canada (St. Constant, Quebec, Canada). The use of these animals was reviewed and approved by Animal Care Committee at DRES. Care and handling of the mice followed guidelines set out by the Canadian Council on Animal Care. Virus was administered to the mice by an intranasal (IN) route. The volume of inoculum used was 50 μL, containing approximately 1×10^4 Plaque Forming Units. For IN administration, mice were anaesthetized with sodium pentobarbital (50 mg/kg body weight, given intraperitoneal - IP). When the animals were unconscious, they were carefully supported by hands with their nose up and the virus suspension in PBS gently applied with a micropipette into the nostrils. The applied volume was naturally inhaled into the lungs. Infected animals were observed daily, for up to 14 days postinfection.

Results

Expression of Structural Genes of WEE

In vitro transcription and translation of the insert using TNT T7 rabbit reticulysate system with or without added canine microsomes, demonstrated synthesis of ^{35}S-methionine-labelled proteins of the correct size as indicated by western blot and mmunoprecipitation with monoclonal antibodies to the NC, E1 and E2 proteins (Long *et al.*, 2000). Similarly, the construct pVHX-6 was also demonstrated to produce the correct MW proteins as determined by *in vitro* transcription/translation. The level of expression for pVHX-6 was significantly higher then for pCXH-3 (Fig 1). As for pCXH-3, pVHX-6 lysates also reacted with monoclonal antibodies to WEE in western blot and immunoprecipitation analysis (data not shown). Expression of the insert from the CMV promoter was accomplished by transfection of the pCXH-3 plasmid into either Vero or CHO K1 cells. Cells expressing the E1 or E2 proteins were detected through the use of specific E1 or E2 monoclonal antibodies to WEE (Long *et al.*, 2000), followed by histochemical staining with the HRP substrate, Tru-Blue as demonstrated in Fig. 2a. The control cells transfected with pCI alone showed no staining (Fig. 2b), thus, demonstrating the fidelity of the proteins translated and processed from the cloned 26S region by mammalian cells.

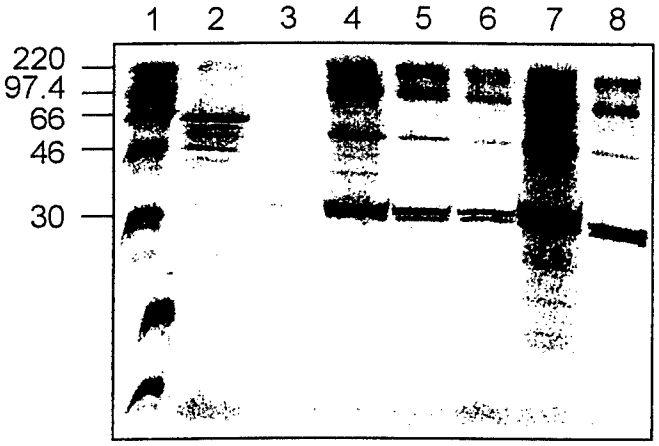

Figure 1. In vitro transcription and translation of WEE expression vectors. *Qiagen purified plasmid preparations containing the WEE 26S insert were expressed* in vitro *using the TNT system and [^{35}S]-methionine labeling. Three µL aliquots of each sample were run by SDS-PAGE on a 12% gel. Lane: 1) Rainbow ^{14}C-labelled marker; 2) Luciferase translation control; 3) pVAX; 4) pVHX-6; 5) pCXH-3; 6) pcDWXH-7; 7) pcDWHX-45; 8) pXTR2-4.*

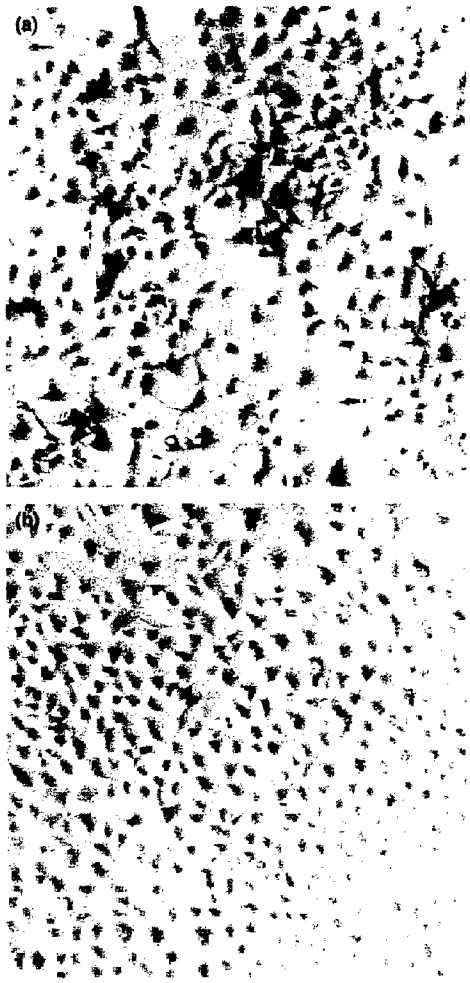

Figure 2. Expression of WEE structural genes in cell culture. *One μg of plasmid DNA was complexed with liposome and transfected into Vero cell monolayers. Expression of WEE antigen was detected with a monoclonal antibody to the WEE E1 protein, 11D2. a. pCXH-3; b. pCI (control plasmid).*

Protection Against WEE Infection Using DNA Immunization

The different strains of WEE were shown to vary in their virulence in BALB/c mice when an IN route of infectivity was used. When similar amounts of WEE were given intranasally to BALB/c mice, time to death was between 4 to 8 days. The California and Fleming strains were the most virulent (Fig. 3), with a time to death of 4 days. CBA 87 was intermediate, while B11 and 71V-1658 were the least virulent. IP administration of the virus did not kill adult mice (data not shown). The Fleming strain was chosen as the challenge strain for subsequent protection studies. The dose of WEE Fleming strain (challenge strain) was 1.25 x 10^4 PFU for 100% killing via an intranasal route of infection.

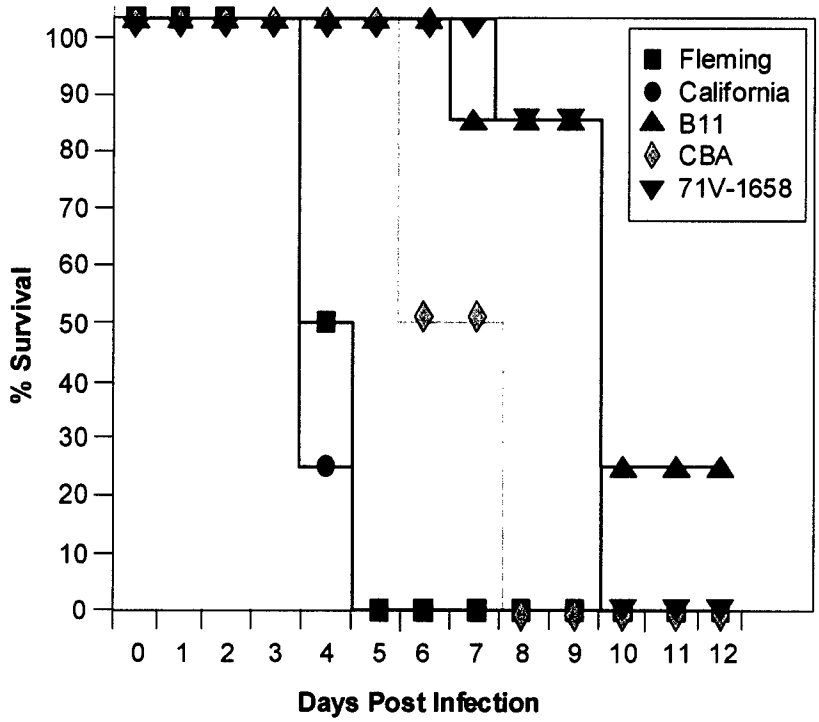

Figure 3. WEE mouse infectivity model. *Groups of 4 mice were inoculated intranasally with 50 µL of different strains of WEE virus (approximately 10^4 PFU). The mice were monitored for 12 days, and the % survival graphed.*

Intramuscular administration of pCXH-3 showed variable protection, using up to three doses of 50 µg, followed by challenge 30 to 90 days after the final dose (data not shown). Intramuscular administration did result in an increase in antibody titre to WEE as determined by ELISA, and partial protection was observed (0 - 50%) (data not shown). The pCXH-3 DNA protected mice when delivered ballistically and using pCI as a control DNA. When two doses of pCXH-3 was given, protection of 50% was demonstrated as compared to no protection for pCI or a single dose of pCXH-3 (Fig. 4). Preliminary studies examining protection using the pVHX-6 vector the Gene Gun and ballistic delivery were promising. Mice were given four doses (2 x 1.25 ug DNA) every two week, and then challenged 3 week after the final booster. With the pVHX-6 vector, one mouse succumbed immediately to the effects of the sodium pentabarbital (anaesthetic). The remaining three mice showed no signs of coming down with a WEE infection, and remained completely healthy (Fig. 5). Of the four pVAX control mice, all showed signs of WEE infection, and two of the four mice died, while two did recover. When the number of doses was reduced to three (2 weeks apart), 3 of 8 mice survived (38 %) as compared with 0/8 pVAX controls.

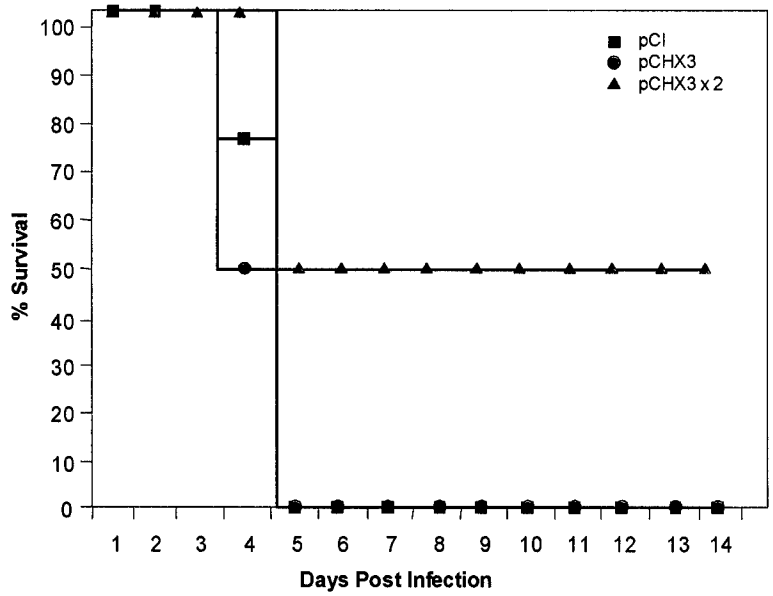

Figure 4. Protection using ballistic delivery of pCXH-3. *Groups of 4 mice were immunized with one or two doses (2 x 1.25 µg) of either pCI control plasmid or pCXH-3 (expressing the WEE structural genes). The interval between boosters (2 doses) or challenge was 3 weeks. The mice were challenged intranasally with 50 µL of WEE Fleming (1.25 x 10^4 PFU). The mice were monitored for 12 days and the % survival graphed.*

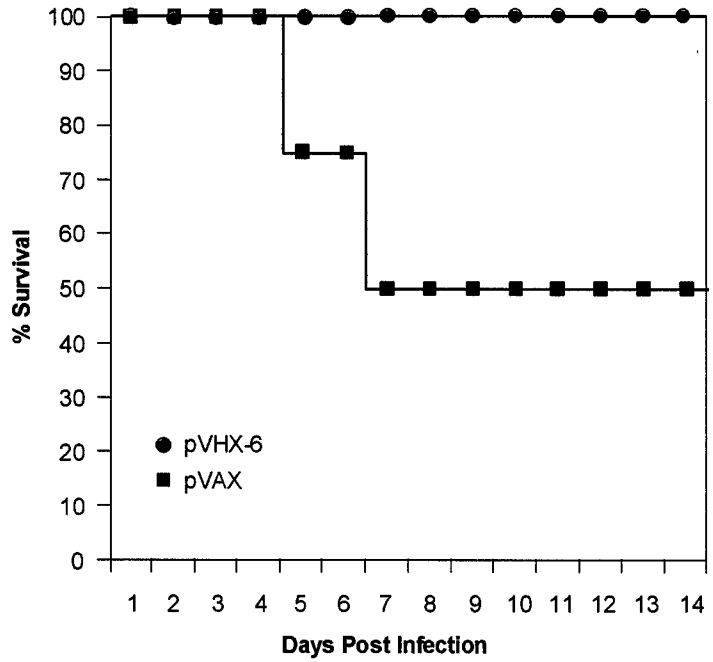

Figure 5. Protection using ballistic delivery of pVHX-6. *Groups of 4 mice were immunized with four doses (2 x 1.25 µg) of pVAX control plasmid or pVXH-6 (expresses the WEE structural genes). The interval between boosters or challenge was 2 weeks. The mice were challenged intranasally with 50 µL of WEE Fleming (1.25 x 10^4 PFU). The mice were monitored for 14 days and the % survival graphed.*

Discussion

The 26S region of 71V-1658 was placed under the control of the CMV promoter of pCI. To test for functional expression of the pCXH-3 pVHX-6 vectors, two methods were employed. A cell-free transcription/translation system yielded the expected sized products as determined by SDS-PAGE. Luciferase was used as a transcription/translation control (Fig. 1, lane 2), and the pVAX as a negative control (Fig. 1, lane 3). Using this system, the pVHX-6 and its parental vector, pcDWHX-45 (Fig. 1, lanes 4 and 7, respectively), expressed to higher levels than pCXH-3 and related constructs (Fig. 1, lanes 5, 6 and 8, respectively). To test for a functional product in cell culture, the pCXH-3 vector was transiently transfected into Vero cells. WEE proteins were detected on/in the cells using specific monoclonal antibodies to both the E1 (Fig. 3a) and E2 proteins (data not shown).

The WEE mouse protection model demonstrates the virulence of WEE as a potential BW agent. When the virus was given IP, no infectivity of adult mice was noted. However, when the same virus was given IN, a lethal model was developed, where the mice rapidly succumb to encephalitis. Strain differences in virulence were noted when examining these five strains of WEE. Additional strains of WEE have been acquired, and a more complete study of this issue will be undertaken, including the cloning and sequencing of the 26S region of Fleming and other selected strains.

The use of pCXH-3 in DNA immunization experiments indicated that the construct could partially protect against WEE intranasal challenge using ballistic delivery. Intramusuclar and intranasal delivery of pCXH-3 DNA yielded little to no protection (data not shown) with and without liposome encapsulation under the conditions used. Ballistic delivery of pCXH-3 did yield up to 50% protection (Fig. 4), using 2 doses. Mice immunized with the pCI control plasmid did not show any signs of protection in this study.

Expression of the WEE structural proteins in the pCI-based vector, pCXH-3, gave moderate to poor levels of expression *in vitro*, using the TNT™ expression kit. A new vector, pVAX (Invitrogen), designed for DNA immunization, but lacking the intron found in the pCI vector, was used to express the WEE structural genes. Initial restriction mapping of pCXH-3 indicated the plasmid was the expected size, but later analysis indicated the possibility of an extra 4 kb fragment being present (data not shown). The WEE structural proteins were cloned and expressed in pVHX-6, indicating the correct sized proteins by SDS-PAGE, and producing higher levels of WEE product *in vitro* (Fig. 1). Preliminary results with pVHX-6 indicated it could completely protect mice against an intranasal challenge of WEE. While 50% of the pVAX mice did survive, they all demonstrated at least moderate to severe infection with WEE. It is possible that pVAX contains CpG motifs that show some protective effect, through a well characterized nonspecific adjuvant like action (Kreig *et al*, 1995, Kreig *et al*, 1998). However, there was a dramatic difference between the pVAX and the pVHX-6 in the protection afforded the two groups of mice. The vector, pCI, did not demonstrate an adjuvant effect when used as a control with pCXH-3. This could be due to the lower number of doses used in the pCXH-3 vaccine study, or a difference in the base vector sequence. In a separate study involving a pCI-based DNA vaccine against influenza A virus, a low level of protection was achieved with pCI vector alone (Wong et al, 2001b). Further studies will be undertaken to

define the dose response profile of the vaccine, potential CpG adjuvant effects, as well as the use of encapsulation for enhancing delivery.

The plasmids, pCXH-3 and pVHX-6 show promise as vaccine candidates for WEE. This is especially important for protection against an aerosol challenge of WEE, an event that would be envisioned in a potential biological warfare attack using WEE as a BW agent. This agent is proving difficult to protect against if delivered aerosolly, as it is thought that the virus infects the nasal membranes, and migrates up the nerves directly into the brain. Recent cross-neutralization data (data not shown) indicates that antibodies to the Fleming strain of WEE do not neutralize the B11, CBA87 and the 71V-1658 strains of WEE. Construction of a second WEE vector with the Fleming 26S region would likely afford greater cross-protection results for all WEE strains, as our original assumption that a single strain of WEE can cross-protect against all WEE isolates may not hold true. New cross-neutralization and cross-protection studies will be carried out to investigate this possibility. The research is applicable to VEE and EEE, as these viruses can also cause encephalitis following a similar route of infection (equines and potentially man). Construction of a DNA vaccine to VEE 1AB is underway thanks to the kind gift of the V3000 plasmid clone from Dr. Jonathan Smith (formerly of the Virology Division, USAMRIID).

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List of symbols/abbreviations/acronyms/initialisms

ATCC	American Type Culture Collection
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
DND	Department of National Defence
DRES	Defence Research Establishment Suffield
EEE	Eastern equine encephalitis virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IM	Intramuscular
IN	Intranasal
IP	Intraperitoneal
LPS	Lipopolysaccharide
NTR	Nontranslated terminal region
PBS	Phosphate-buffered saline
RNA	Ribonucleic acid
SIN	Sindbis virus
VEE	Venezuelan equine encephalitis virus
WEE	Western equine encephalitis virus

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Previously, the complete genome of western equine encephalitis virus had been cloned and sequenced. This paper describes mammalian expression vectors pCXH-3 and pVHX-6, in which expression of the structural genes of western equine encephalitis virus have been placed under the control of the mammalian CMV promoter. When pCXH-3 or pVHX-6 is expressed using a cell-free transcription/translation system, *in vitro*, authentic structural proteins of western equine encephalitis virus are produced as verified by reactivity with monoclonal antibodies developed to western equine encephalitis virus. These vectors can also be complexed with liposomes and administered to mammalian cell culture. The viral envelope proteins were functionally expressed, as determined by histochemical staining with monoclonal antibodies which recognize WEE antigens. In addition, when pCXH-3 or pVHX-6 is administered intraepidermally and intramuscularly to mice, a protective immune response is induced. Immunized mice had a significantly reduced risk of infection, against a subsequent intranasal challenge with western equine encephalitis virus. Development of a DNA vaccine to western equine encephalitis virus is promising. In a similar manner, DNA vaccines to related alphaviruses (Venezuelan and eastern equine encephalitis viruses) could also be developed.

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DNA vaccine, DNA immunization, alphavirus, western equine encephalitis

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